Isolation and characterization of derhamnosylating alkaline α-L-rhamnosidase from *Aspergillus niger*

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Abstract

α-L-Rhamnosidases are ubiquitous enzymes responsible for derhamnosylation of α-L-rhamnose moiety from a variety of glycoconjugates including rutin, naringin, hesperidin, quercitrin, terpenyl glycosides and numerous other natural glycosides. An α-L-rhamnosidase-secreting fungal strain was isolated from a soil sample collected at a local fruit market. This strain was initially identified as Aspergillus sp. through lactophenol cotton blue staining and later confirmed as Aspergillus niger via ITS gene sequencing. The rhamnosidase activity of the fungal strain was screened on modified Czapek-Dox agar medium supplemented with naringin.

Enzyme production was assessed in liquid culture medium containing naringin, rutin and hesperidin as inducers. The optimal pH and temperature for maximum catalytic efficiency of the crude α-L-rhamnosidase were found to be pH 10.0 and 70°C respectively. The Michaelis constant (Km) and maximum velocity (Vmax) for the crude enzyme towards naringin were determined to be 0.042 mM and 0.062 µmol min^{-1} ml^{-1} respectively.

Keywords: α-L-Rhamnosidase, L-rhamnose, Glycosides, *Aspergillus niger*, Naringin.

Introduction

Glycoside hydrolases (GHs), also termed glycosidases, constitute a diverse class of enzymes that catalyse the hydrolytic cleavage of glycosidic bonds within carbohydrates and other glycoconjugates[5]. Glycoside hydrolases (GHs) are classified based on sequence similarity, structure and catalytic mechanisms. Currently, more than 170 GH families have been identified, reflecting their diverse functions and evolutionary origins[1]. α-L-Rhamnosidase [EC 3.2.1.40], classified in the CAZy database under the glycoside hydrolase families GH13, GH78 and GH106, is a specialized enzyme that hydrolyzes terminal α-L-rhamnose residues from the non-reducing ends of a wide range of substrates[13].

This enzyme is prevalent in nature, being found in microorganisms, plants and animals where it plays a critical role in the removal of α-L-rhamnose moieties from glycoconjugates such as rutin, naringin, hesperidin, quercitrin, terpenyl glycosides and various other naturally derived glycosides[26,28,31,32]. These natural glycosides, widely distributed in plant sources, often exhibit limited bioavailability, necessitating enzymatic modifications to enhance their biological activity and applicability. This enzyme has emerged as biotechnologically significant due to its diverse applications across multiple industries. In the food and beverages, it is employed for debittering citrus fruit juices by hydrolyzing naringin to produce prunin[12,14,23].

Similarly, it is used to enhance wine aromas by enzymatically hydrolyzing terpenyl glycosides containing α-L-rhamnose, by releasing aromatic aglycones[18], removing hesperidin molecules from orange juices[21]. In pharmaceutical applications, it is used to generate bioactive compounds with enhanced pharmacological properties. For example, prunin, derived from naringin, exhibits potent anti-inflammatory activity and antiviral effects against both DNA and RNA viruses[6]. Quercetin, produced through the derhamnosylation of quercitrin, demonstrates anticarcinogenic, antioxidative, anti-inflammatory, vasodilating and antiaggregatory properties, while hesperetin, obtained from hesperidin, has shown anticarcinogenic effects in preclinical studies[7].

Additionally, it has been involved in the hydrolysis of L-rhamnose-containing steroids such as desglucoruscin, ginsenosides-Rg2 and diosgenin, whose modified forms are of clinical importance due to their therapeutic potential. Derhamnosylation of rutin (quercetin-3-O-rutinoside) yields isoquercitrin (quercetin-3-O-β-glucoside), an antithrombotic drug with anti-inflammatory, antiviral and other pharmacological benefits for treating myocardial ischemia, cerebral hypoxia and ischemic diseases. Icaritin (a clinically important and safer drug precursor for treating cancers) is produced from epimedin C by enzymatic action of α-L-rhamnosidase[24].

In this study, the reported crude α-L-rhamnosidase enzyme, harvested from *Aspergillus niger*, is thermostable and exhibits maximal activity in an alkaline pH range which can be suitable for conversion of naringin to prunin, rutin to isoquercitrin, as bioavailable compound with significant pharmaceutical values.

Material and Methods

Materials: Naringin, rutin and hesperidin were procured from Sigma Chemical Company, St. Louis, (USA). Calcium chloride, magnesium sulphate, potassium phosphate, manganous sulphate, ammonium tartarate, sodium acetate, sodium carbonate and sodium bicarbonate were from Himedia, Mumbai, (India). Citric acid, tartaric acid, acetic acid and other chemicals were purchased either from SRL Chem. Ltd. or from Qualigens.

Isolation and Screening of α-L-rhamnosidase producing fungal strain: The fungal isolate was cultivated in our laboratory from soil sample collected at a local fruit market, specifically from an area where rotten citrus fruits were dumped. This location was chosen as the source of the sample due to the likelihood of fungal growth in nutrient-rich, decaying organic matter. For the isolation process, the soil sample was streaked onto a Petri plate containing potato dextrose agar medium using the streaking method[17]. The isolated strain was preserved on slants of potato dextrose agar (PDA) medium, which was subcultured fortnightly to ensure viability. The isolated fungal strain was screened for their potential to produce α-L-rhamnosidase by qualitative Naringin agar plate assay method.

For this purpose, a mycelial plug was transferred onto a modified Czapek dox agar medium (MCDA) supplemented with 0.5 % (w/v) naringin instead of sucrose as soul carbon source. The Naringin agar medium (NAM) was prepared with composition of $NaNO_3$ 2.0 g/L, $MgSO_4$ 0.5 g/L, K_2HPO_4 1.0 g/L, $FeSO_4$ 0.01 g/L, KCl 0.5 g/L, agar 15.0 g/L and naringin (0.5% w/v). All Petri plates were incubated at a controlled temperature of 25°C in biological oxygen demand (BOD) incubator to facilitate fungal growth. The incubation process was carried out for a duration of five days and the halo zone around colonies was evaluated.

Identification of isolated fungal strain: Molecular identification of isolated fungal strain was carried out using the 18S rRNA and internal transcribed spacer (ITS) genes. The resultant sequences were subjected to BLASTn search with default parameters at the NCBI GenBank database to identify closely related fungal species. Sequences with 100% identity to *Aspergillus* species were selected for further analysis. These sequences were downloaded in FASTA format and aligned using the *CLUSTALW* tool embedded in MEGA11 software[20]. The evolutionary relationship was reconstructed following the neighbor-joining (NJ) approach[16,19]. The analysis included a total of 44 nucleotide sequences and the final dataset comprised of 1,558 positions for the evolutionary comparison.

Secretion of α-L-rhamnosidase: The secretion of a-L-rhamnosidase by the isolated fungal strain was investigated using a method described in the available literature[28]. The liquid culture medium consisted of 1000 mL Milli-Q water containing $CaCl_2$ (1.0 g), $MnSO_4$ (1.0 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), $FeSO_4.7H_2O$ (0.1 g), KH_2PO_4 (20.0 g), $CuSO_4 \cdot 5H_2O$ (0.1 g), $MgSO_4 \cdot 7H_2O$ (3.0 g), $N(CH_2COONa)_3$ (1.5 g), H_3BO_3 (10.0 mg), sucrose (40.0 g) and ammonium tartrate (8.0 g). Spores from a fresh agar plate were aseptically inoculated into 80 mL of sterilized liquid culture medium in 250 mL Erlenmeyer culture flasks, which were additionally enriched with 0.5 percent naringin or rutin or hesperidin as inducer[30].

The flasks were incubated at 25°C in a BOD incubator under static conditions. At 24-hour intervals, one-milliliter aliquots of the liquid culture medium were taken, filtered through 0.22 µm Millex syringe filters and subsequently analysed for activity of α-L-rhamnosidase enzyme.

Assay of α-L-rhamnosidase activity: The enzymatic activity of α-L-rhamnosidase was assessed using naringin as substrate based on the Davis method with minor modifications[2]. The enzyme activity was assessed at both acidic and basic pH conditions. The reaction mixture consisted of 950 µL of 0.86 mM naringin dissolved in either 0.1 M sodium acetate-acetic acid buffer (pH 5) or 0.1 M sodium bicarbonate-carbonate buffer (pH 9), maintained at 50°C. A 50 µL aliquot of enzyme extract was added to initiate the reaction. At 0 and 15 minutes, 100 µL aliquots of the reaction mixture were withdrawn. Each aliquot was immediately mixed with 2.5 mL of 90% diethylene glycol (DEG), followed by subsequent addition of 100 µL of 4N NaOH to promote color development. The tubes were then incubated at ambient temperature for at least 10 minutes to allow the yellow color to fully develop.

Absorbance at 420 nm (OD420) was measured spectrophotometrically using a Shimadzu 1900i double-beam UV-Vis spectrophotometer. For the non-enzymatic control, the crude enzyme extract was filtered through an Amicon ultra centrifugal filter (10 kDa MWCO) and heat-treated at 100°C for 30 minutes to deactivate the enzyme. Naringin hydrolysis was then measured using this heat-treated filtrate. Enzymatic activity was determined by subtracting the absorbance values from the non-enzymatic control from those of the enzymatic reaction, ensuring an accurate measurement of the enzyme's catalytic activity. The minimum measurable change for absorbance readings was 0.001 absorbance units.

The absorbance readings were calculated naringin concentrations using a calibration curve specific to naringin. The rate of naringin hydrolysis was evaluated from the graph plotting the reduction in naringin concentration over time. Enzyme activity (1 IU) is defined as the quantity of enzyme required to hydrolyze 1 µmol of naringin per minute under defined assay conditions.

Kinetic characterization of α-L-rhamnosidase: The enzymatic properties of the crude enzyme were characterized by utilizing naringin as substrate, following the naringin hydrolysis method as described previously[2]. To estimate the optimal pH, enzyme activity was measured over a pH range from 2 to 13 at 50°C, using the following 0.1 M buffers: citric acid–NaH_2PO_4 (McIlvaine buffer, pH 2–7), Clark and Lubs solution (pH 8–10), Na_2HPO_4–NaOH buffer (pH 11–12) and KCl–NaOH buffer (pH 13).

For the temperature optima study, enzyme activity was assayed across a temperature range of 30°C to 100°C, using a 0.1 M sodium carbonate buffer (pH 10.0). Steady-state enzyme velocity was measured using naringin as the substrate (0.025 to 1.5 mM) in 0.1 M sodium carbonate-bicarbonate buffer (pH 10.0) at 70°C.

Results and Discussion

Isolation and Identification of α-L-rhamnosidase producing fungal strain: The black fungal colonies were initially isolated on potato dextrose agar (PDA) plates (Fig. 1A) and tentatively identified as *Aspergillus species* based on their morphological characteristics, observed under lactophenol cotton blue staining (Fig. 1B). To confirm the identification, molecular identification was performed through 18S rRNA gene sequencing, coupled with evaluation of the internal transcribed spacer (ITS) region. This molecular approach provided precise taxonomic classification, conclusively identifying the strain as *Aspergillus niger.* The fungal strain cultured on modified Czapek Dox agar plates supplemented with 0.5% (w/v) naringin, exhibited a prominent halo zone around the colonies, indicating enzymatic activity (Fig. 1C).

The clear zone, or decoloration, surrounding the colonies suggests the potential production of α-L-rhamnosidase, as it correlates with the hydrolysis of naringin. This observation implies that the enzyme is being produced in response to the substrate. The production of α-L-rhamnosidase is likely triggered by the induction of rha gene expression, activated by rhamnose-containing compounds, such as naringin, which serves both as a sole carbon source and an inducer of enzyme production[11]. This induction mechanism highlights the adaptability of fungal strains in utilizing specific carbon sources and their ability to regulate enzyme production in response to substrate availability.

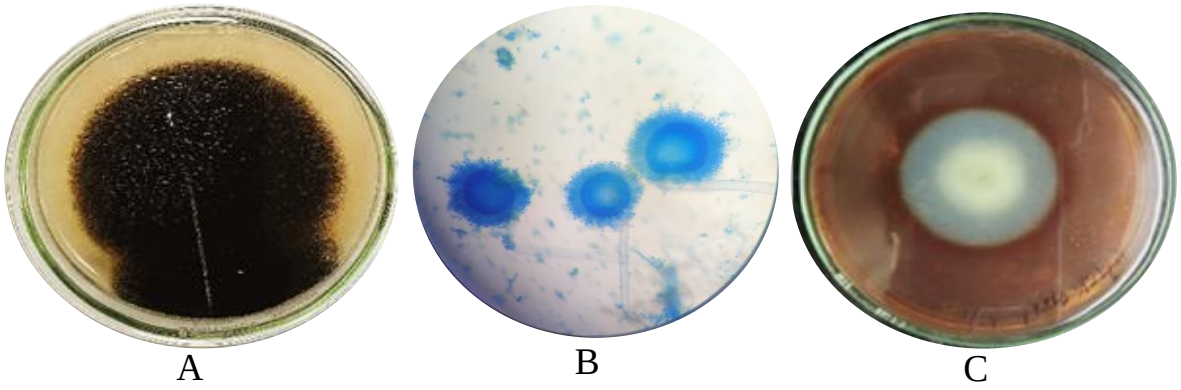


Figure 1: (A) Isolation of fungal strain from soil on PDA medium, (B) Morphological identification of isolated fungal strain by lactophenol cotton blue staining method, (C) Showing halo zone formation around the colony due to secretion of α-L-rhamnosidase on agar plate supplemented with naringin.

Figure 2: Neighbor-joining (NJ) tree analysis showing the Phylogenetic relation of *Aspergillus niger.* The tree has a total of 44 nucleotides sequences in which red marked fungal sequences came from the present study, 42 as reference sequences of closely related species retrieve from NCBI database. and rooted with sequence from *Bacillus subtilis* (NR112116) as an outgroup

Phylogenetic tree analysis: The phylogenetic tree was visualized within MEGA 11, where the branches were annotated with support values, providing insights into the evolutionary relationships of *Aspergillus niger* and its related species (Fig. 2). This approach, combining the 18S rRNA and ITS gene sequences, allows for a thorough insight into the genetic diversity and taxonomic position of *Aspergillus niger* within the *Aspergillus* genus.

Secretion of α-L-rhamnosidase: The production and secretion of the enzyme α-L-rhamnosidase from *Aspergillus niger* were systematically investigated under controlled laboratory conditions using a liquid culture medium supplemented with various flavonoid inducers, specifically naringin, rutin and hesperidin. The principal objective of this study was to evaluate the enzymatic activity of α-L-rhamnosidase and to optimize the culture conditions to maximize enzyme yield. The extracellular α-L-rhamnosidase demonstrated distinct peak activity profiles depending on the flavonoid inducer present in the culture medium. Each of the three inducers was added at a consistent concentration of 0.5% and enzyme activity was monitored over a period of eight days post-inoculation.

Notably, the optimal enzymatic activity occurred on different days depending on the flavonoid substrate used. Specifically, maximal α-L-rhamnosidase activity was recorded on the 3rd day for rutin, on the 4th day for naringin and on the 5th day for hesperidin (Fig. 3). For crude enzyme preparation, the fungal strain was cultivated in liquid culture medium amended with 0.5% naringin as inducer at 25°C for 96 hours in biological oxygen demand (BOD) incubator under stationary environment. These results suggest that the induction of α-L-rhamnosidase synthesis by *Aspergillus niger* is highly responsive to the type of flavonoid present in the growth medium, with each flavonoid influencing both the timing and magnitude of enzyme production. These findings highlight the differential temporal production of α-L-rhamnosidase in response to various flavonoid inducers, providing key insights into the optimization of production conditions for this enzyme. Further investigations into the mechanistic pathways underlying these variations could enhance the understanding of isolated *Aspergillus niger* metabolic responses to flavonoid inducers and could contribute to the development of more efficient production systems for α-L-rhamnosidase.

Steady-state kinetics of α-L-rhamnosidase: The study findings show that the crude enzyme activity varies when the pH values changes. The crude α-L-rhamnosidase, using naringin as a substrate, exhibited its optimal enzymatic activity (Figs. 4 and 5). The enzyme exhibited relatively low activity within the acidic pH range. However, a notable increase in activity was noticed in the alkaline range, with a sharp rise occurring after pH 8. The activity peaked at pH 10 but declined noticeably beyond this point, showing reduced activity at pH 12 (Fig. 4).

This pattern suggests that the enzyme functions optimally in a moderately alkaline environment, with diminished efficiency under highly acidic or strongly alkaline conditions. A significant number of α-L-rhamnosidases documented to date exhibit pH optima in either acidic[8,9,11] pH range, near neutral[3,27] or in the basic[15,29,30] pH range. Optimal activity of the crude alpha-L-rhamnosidase was observed under alkaline pH conditions, indicating its potential for the hydrolysis of naringin to prunin and rutin to isoquercitrin and both are bioavailable and bioactive compounds.

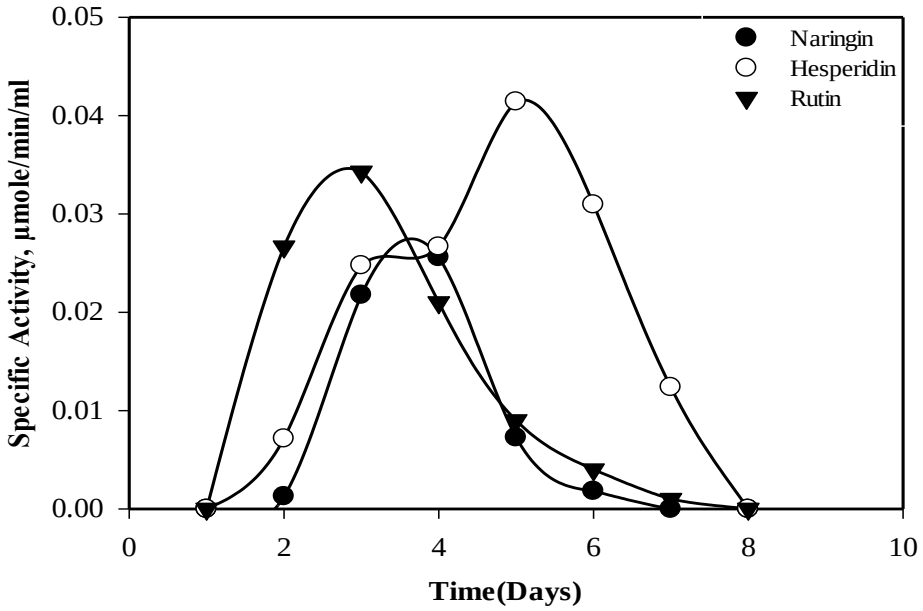


Figure 3: Secretion of α-L-rhamnosidase in the liquid culture medium of *Aspergillus niger* containing 0.5% (w/v) of hesperidin (○), rutin (▼) and naringin (●).

The temperature activity profile of the crude α-L-rhamnosidase showed a gradual increase in enzymatic activity starting from 30°C, attaining its optimum at 70°C under pH 10.0 conditions (Fig. 5). Beyond 70°C, the activity declined significantly, with reduced activity observed at 90°C, likely due to loss of activity at extreme temperature.

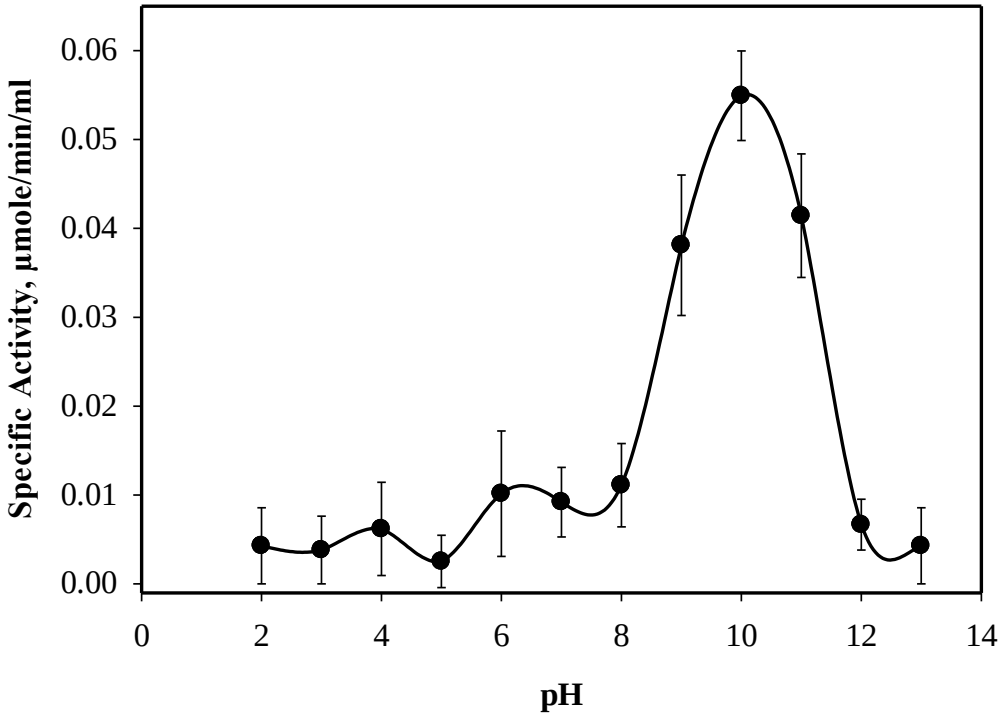


Figure 4: Effects of pH on the activity of the enzyme. The enzyme assay solution 1.0 mL contained 0.86 mM naringin, 0.05 mL crude enzyme extract in 0.1 M citric acid–NaH_2PO_4 (McIlvaine buffer pH 2-7), Clarks and Lubs solution (pH 8–10), Na_2HPO_4- NaOH buffer (11-12) and KCl-NaOH buffer (pH 13) at 50ºC.

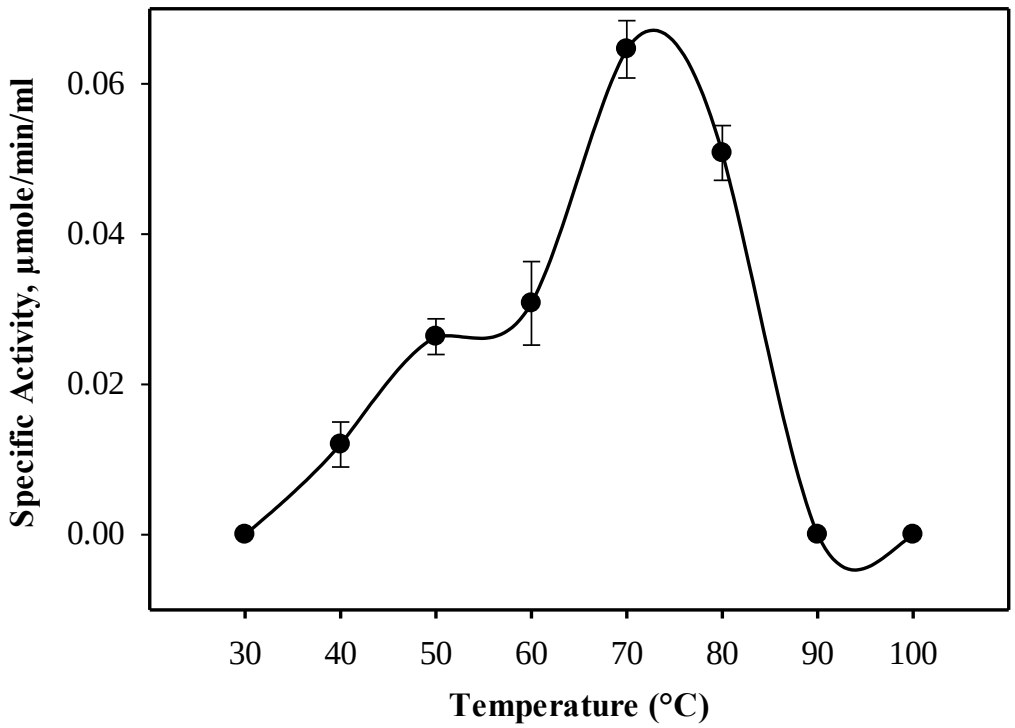
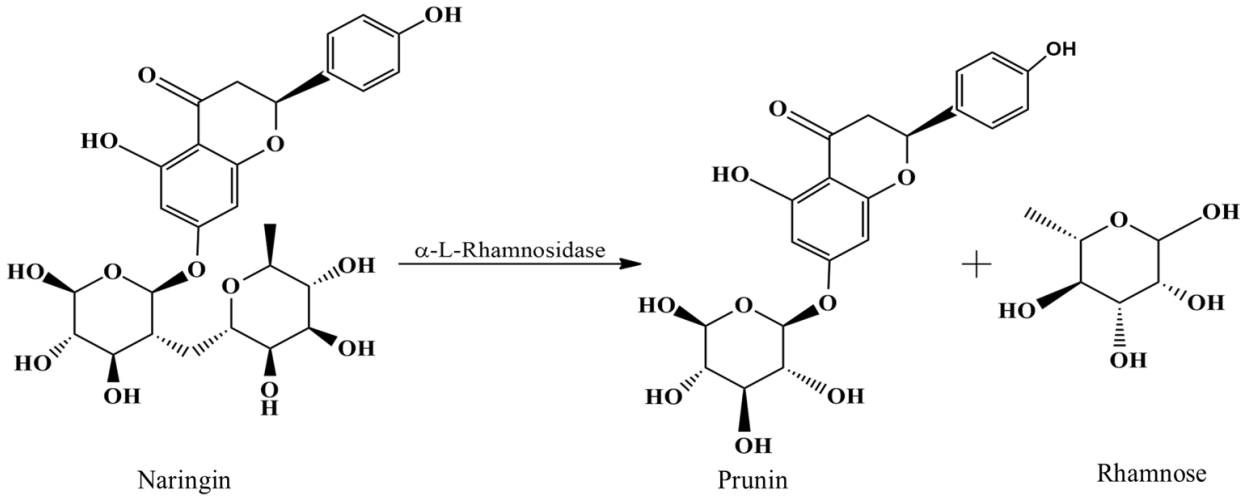


Figure 5: Effect of temperature on the activity of the crude enzyme. The assay solution 1.0 mL contained 0.86mM naringin, 0.05 mL crude enzyme extract in 0.1 M sodium carbonate-bicarbonate buffer pH 10 at varying temperatures (30-100ºC)

The α-L-rhamnosidase Km and Vmax values from *Aspergillus niger* were calculated by measuring steady-state reaction velocities at different concentrations of naringin and drawing Michaelis–Menten plot (Fig. 6). The estimated Km and Vmax were found to 0.042 mM and 0.062 µmole min^{-1} ml^{-1} respectively. The obtained kinetic parameters are in accordance to previous reports. The Km values for α-L-rhamnosidase purified from *Dictyoglomus thermophilum*[4], from *Novosphingobium sp. PP1Y*[22], from *Aspergillus awamori* MTCC-2879[25] and from *Penicillium griseoroseum* MTCC-9224[26] have been found to be 0.05, 015, 0.62 and 0.65mM respectively. The Vmax values for purified α-L-rhamnosidase from *Aspergillus awamori* MTCC-2879[27], from *Penicillium griseoroseum* MTCC-9224 and from *Aspergillus niger* JMU-TS528[10] were reported as 15.8 U/mg, 20.4 U/mg and 0.460mmol min^{-1} respectively.

Conclusion

This study led to the successful isolation of *Aspergillus niger* from a soil sample collected at a site with decaying citrus fruits. The isolated strain was found to produce an α-L-rhamnosidase enzyme with remarkable enzymatic properties. The enzyme exhibited high thermostability and retained its catalytic efficiency over a broad range of alkaline pH, suggesting its potential for industrial applications that demand both elevated temperatures and alkaline conditions. These characteristics make it particularly suitable for processes such as the biotransformation of flavonoids and the production of bioactive compounds. Notably, the enzyme could facilitate the conversion of naringin to prunin and rutin to isoquercitrin, two key reactions in the synthesis of functional food ingredients and pharmaceutical intermediates.



Scheme 1: Hydrolysis of naringin to prunin by α-L-rhamnosidase

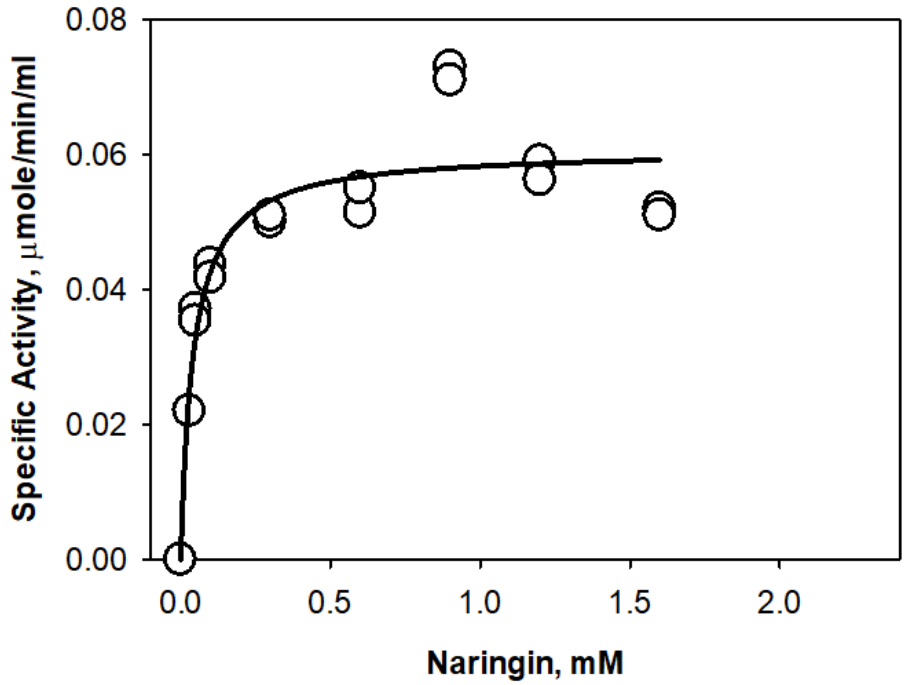


Figure 6: Michealis–Menten plots of the crude enzyme using naringin as the substrate. The assay solution 1.0 mL contained 0.025–1.5mM naringin, 0.05 mL of crude enzyme in 0.1 m sodium carbonate buffer pH 10 at 70°C

The thermostable and alkaline-stable nature of this α-L-rhamnosidase positions it as a promising candidate for pharmaceutical applications where enzymes are often required to operate under harsh conditions. Moreover, the enzyme's robustness under high temperature and alkaline pH conditions enhances its industrial relevance, particularly in the synthesis of bioactive molecules and in the biocatalysis of glycosylated compounds. These findings open the door to further exploration of *Aspergillus niger* as a viable source of enzymes for various biotechnological and industrial applications.

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References

1. Amin K., Tranchimand S., Benvegnu T., Abdel-Razzak Z. and Chamieh H., Glycoside Hydrolases and Glycosyltransferases from Hyperthermophilic Archaea: Insights on Their Characteristics and Applications in Biotechnology, *Biomolecules*, **11**, 1557 **(2021)**

2. Davis W.B., Determination of Flavanones in Citrus Fruits, *Analytical Chemistry*, **19**, 476-8 **(1947)**

3. Ge L., Liu Y., Zhou F., Zhan L. and Zhao L., Heterologous Expression and Characterization of a Thermostable & alpha;-L-Rhamnosidase from Thermoclostridium stercorarium subsp. thermolacticum DSM 2910 and Its Application in the Biotransformation of Rutin, *J. Microbiol. Biotechnol.*, **33**, 1521-30 **(2023)**

4. Guillotin L. et al, Biochemical Characterization of the α-l-Rhamnosidase DtRha from Dictyoglomus thermophilum: Application to the Selective Derhamnosylation of Natural Flavonoids, *ACS Omega*, **4**, 1916-22 **(2019)**

5. Henrissat B. and Davies G., Structural and sequence-based classification of glycoside hydrolases, *Curr Opin Struct Biol*, **7**, 637-44 **(1997)**

6. Kaul T.N., Middleton E. Jr. and Ogra P.L., Antiviral effect of flavonoids on human viruses, *J Med Virol*, **15**, 71-9 **(1985)**

7. Krogholm K.S., Bredsdorff L., Knuthsen P., Haraldsdóttir J. and Rasmussen S.E., Relative bioavailability of the flavonoids quercetin, hesperetin and naringenin given simultaneously through diet, *Eur J Clin Nutr*, **64**, 432-5 **(2010)**

8. Li L. et al, Heterologous Expression and Characterization of a New Clade of Aspergillus α-L-Rhamnosidase Suitable for Citrus Juice Processing, *Journal of Agricultural and Food Chemistry*, **67**, 2926-35 **(2019)**

9. Li L. et al, Expression and biochemical characterization of recombinant α-l-rhamnosidase r-Rha1 from Aspergillus niger JMU-TS528, *International Journal of Biological Macromolecules*, **85**, 391-9 **(2016)**

10. Li L.J. et al, Preparation of isoquercitrin by biotransformation of rutin using α-L-rhamnosidase from Aspergillus niger JMU-TS528 and HSCCC purification, *Preparative Biochemistry & Biotechnology*, **50**, 1-9 **(2020)**

11. Manzanares P., van den Broeck H.C., de Graaff L.H. and Visser J., Purification and characterization of two different alpha-L-rhamnosidases, RhaA and RhaB, from *Aspergillus aculeatus*, *Appl Environ Microbiol*, **67**, 2230-4 **(2001)**

12. Olson A.C., Gray G.M. and Guadagni D.G., Naringin bitterness of grapefruit juice debittered with naringinase immobilized in a hollow fiber, *Journal of Food Science*, **44**, 1358-61 **(1979)**

13. Pan L., Zhang Y., Zhang F., Wang Z. and Zheng J., α-L-rhamnosidase: production, properties and applications, *World J Microbiol Biotechnol*, **39**, 191 **(2023)**

14. RoitNer M., Schalkhammer T. and Pittner F., Preparation of prunin with the help of immobilized naringinase pretreated with alkaline buffer, *Applied Biochemistry and Biotechnology*, **9**, 483-8 **(1984)**

15. Rojas N.L., Voget C.E., Hours R.A. and Cavalitto S.F., Purification and characterization of a novel alkaline α-L-rhamnosidase produced by Acrostalagmus luteo albus, *J Ind Microbiol Biotechnol*, **38**, 1515-22 **(2011)**

16. Saitou N. and Nei M., The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Molecular Biology and Evolution*, **4**, 406-25 **(1987)**

17. Seeley H. Jr., Van Demark P.J., and J. J. Lee., Microbes in action: A laboratory manual of microbiology, Fourth ed., W.H. Freeman and Co., New York, NY **(1995)**

18. Spagna G., Barbagallo R.N., Martino A. and Pifferi P.G., A simple method for purifying glycosidases: alpha-l-rhamnopyranosidase from *Aspergillus niger* to increase the aroma of Moscato wine, *Enzyme Microb Technol*, **27**, 522-30 **(2000)**

19. Tamura K., Nei M. and Kumar S., Prospects for inferring very large phylogenies by using the neighbor-joining method, Proceedings of the National Academy of Sciences, **101**, 11030-5 **(2004)**

20. Tamura K., Stecher G. and Kumar S., MEGA11: Molecular Evolutionary Genetics Analysis Version 11, *Mol Biol Evol.*, **38**, 3022-7 **(2021)**

21. Terada Y., Kometani T., Nishimura T., Takii H. and Okada S., Prevention of Hesperidin Crystal Formation in Canned Mandarin Orange Syrup and Clarified Orange Juice by Hesperidin Glycosides, *Food Science and Technology International, Tokyo*, **1**, 29-33 **(1995)**

22. Terry B., Ha J., De Lise F., Mensitieri F., Izzo V. and Sazinsky M.H., The crystal structure and insight into the substrate specificity of the α-L rhamnosidase RHA-P from Novosphingobium sp. PP1Y, *Archives of Biochemistry and Biophysics*, **679**, 108189 **(2020)**

23. Tsen H.Y. and Yu G.K., Limonin and Naringin Removal from Grapefruit Juice with Naringinase Entrapped in Cellulose Triacetate Fibers, *Journal of Food Science*, **56**, 31-4 **(1991)**

24. Xie J., Zhang S., Tong X., Wu T., Pei J. and Zhao L., Biochemical characterization of a novel hyperthermophilic α-l-rhamnosidase from Thermotoga petrophila and its application in production of icaritin from epimedin C with a thermostable β-glucosidase, *Process Biochemistry*, **93**, 115-24 **(2020)**

25. Yadav S., Yadav R.S.S. and Yadav K.D.S., An α-l-rhamnosidase from spergillus awamori MTCC-2879 and its role in debittering of orange juice, *International Journal of Food Science & Technology*, **48**, 927-33 **(2013)**

26. Yadav S., Yadava S. and Yadav K.D., α-l-rhamnosidase selective for rutin to isoquercitrin transformation from Penicillium griseoroseum MTCC-9224, *Bioorg Chem*, **70**, 222-8 **(2017)**

27. Yadav S., Yadava S. and Yadav K.D.S., Purification and characterization of α-l-rhamnosidase from Penicillium corylopholum MTCC-2011, *Process Biochemistry*, **48**, 1348-54 **(2013)**

28. Yadav V., Yadav P.K., Yadav S. and Yadav K.D.S., α-l-Rhamnosidase: A review, *Process Biochemistry*, **45**, 1226-35 **(2010)**

29. Yadav V., Yadav S., Yadav S. and Yadav K., Alpha-L-rhamnosidase from Aspergillus clavato-nanicus MTCC-9611 active at alkaline pH, *Prikladnaia Biokhimiia i Mikrobiologiia*, **48**, 328-33 **(2012)**

30. Yadav V., Yadav S.R., Yadava S. and Yadav K.D.S., α-L-Rhamnosidase from Aspergillus flavus MTCC-9606 isolated from lemon fruit peel, *International Journal of Food Science and Technology*, **46**, 350-7 **(2011)**

31. Zhang T., Yuan W., Li M., Miao M. and Mu W., Purification and characterization of an intracellular α-l-rhamnosidase from a newly isolated strain, Alternaria alternata SK37.001, *Food Chem*, **269**, 63-9 **(2018)**

32. Zverlov V.V., Hertel C., Bronnenmeier K., Hroch A., Kellermann J. and Schwarz W.H., The thermostable alpha-L-rhamnosidase RamA of Clostridium stercorarium: biochemical characterization and primary structure of a bacterial alpha-L-rhamnoside hydrolase, a new type of inverting glycoside hydrolase, *Mol Microbiol*, **35**, 173-9 **(2000)**.

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